provide the following table that illustrates the uniqueness of the monoclonal antibody used in the present invention.

The commercially available monoclonal antibody used in the present invention is derived from a hybridoma clone designated D1G1OVL2 and was prepared using a well defined immunogen composed of the peptide GHRPLDKC (SEQ. ID. NO. 2) conjugated to bovine serum albumin. The resulting monoclonal antibody binds specifically to peptide sequence SEQ. ID. NO. 1 which is unique to the beta chain of the human fibrinogen molecule. The sequence peptide having SEQ. ID. NO. 1 was selected because the present inventors observed that a specific protease associated with certain cancer cells cleaved the fibrinogen beta chain at a site immediately preceding SEQ ID NO 1. Thus, it was theorized that assay specificity would be enhanced if a monoclonal antibody directed to SEQ. ID. NO. 1 was used as the capture antibody.

The Applicants also note that the Okajima et al. reference provided to the Examiner in the Applicant's IDS was of poor quality and does not permit the Examiner to clearly interpret the figures. The figures 1 and 3 show the specificity of the antibodies used in assays of the Okajima et al. reference. Consequently, it is essential that these figures be clearly resolved in order for the Examiner to consider the arguments presented below. Therefore, as a courtesy to the Examiner, a clean copy of the Okajima et al. reference has been attached to this response.

The following chart distinguishes the monoclonal antibody of the present invention from the antibodies found in the cited prior art:

Table 1

Reference	Antibody Designation	Antibody Specificity (Immunogen source)	Distinguishing Differences
Koopman et al.	FDP-14	E_{neo} determinant exposed in the E fragment. The E_{neo} fragment is found on the gamma chain of the fibrinogen molecule.[1]	The monoclonal antibody of the present invention is directed to an epitope at a cleavage site near the N-terminus of the beta chain (SEQ. I.D. NO. 1). SEQ. I.D. NO. 1 is not found in the gamma chain of fibrinogen.[2]
McCulloch, et al.			Uses the Koopman et al assay, therefore the distinguishing characteristics between Koopman and the present invention apply here as well.
Amiral, et al.	Group 1: MABs 9C3, 8B10 and 2C2. Group 2: MABs CX1F2, 15F2, 7G2. Group 3: MABs 14G7, 13F3, 5F7 and 12E3	Group 1: High reactivity with D dimmer and FgDP-D[3] Group 2: Good reactivity with D-dimmer, poor with FgDP-D Group 3: Preferential activity with early FgDP-X and Y, Early FbDP but low or now activity with FgDP-D or D dimmer.	Group 1:Immunogen is derived from a mixture of native, digested proteins. Note that a specific synthetic polypeptide reactively directed against the polypeptide of SEQ ID NO. 1. Group 2: antibodies did not show reactivity against FgDP when used in an immunoassay (see page 449 column two at lines 18-23) The Monoclonal of the

[1] See Plow, E.F. and T.S. Edington. 1975. *A Cleavage-associated Neoantigenic Marker for a γ Chain Site in the NH_2-terminal Aspect of the Fibrinogen Molecule.* The Joun. Biol. Chem. 250, No. 93386-3392. (Reference provided as courtesy to the Examiner.)

[2] The amino acid sequence listing for the complete gamma chain of human fibrinogen has been provided as a courtesy to the Examiner.

[3] Fg = Fibrinogen, Fb = Fibrin, FgDP-D = Fibrinogen degradation product from D dimmer, FbDP = Fibrin degradation product.

			present invention has high affinity for the FgDP antigen. Group 3: antibodies show activity against the early fibrinogen antigens but no or little activity against the FgDP.
Okajima et al	JIF-23 and JIF-25	JIF-23: Not used in an immunoassay. JIF-25: The immunogen was derived from the gamma chain of fibrinogen, specifically aa 86-302.	However, it was used as a capture antibody (see Figure 3 of the Okajima et al. reference). As demonstrated in this Figure (lane 1) the JIF-23 monoclonal is reactive with, i.e. captures, the E domain of fibrinogen FgDP. The monoclonal antibody of the present invention is non-reactive with the E domain of FgDP, therefore, JIF-23 is not the same and the monoclonal used in the present invention. The monoclonal antibody of the present invention is directed at an epitope on the beta chain.

As illustrated in the preceding chart, the specificity of the antibodies used in the prior art can be clearly distinguished from the present invention. Group 1 antibodies used by Amiral et al. do not demonstrate the specificity for the peptide sequence used by the present inventors. The Group 1 antibodies used by Amiral et al. were derived from an animal immunized with a native immunogen mix whereas the immunogen of the present invention was a specific small polypeptide of known specificity. However,

neither D-dimmer fragment nor FgDP-D gragment contains the peptide sequence of GHRPLDK (M. Furlan, 1988, "Structure of Fibrinogen and Fibrin," in Figrinogen, Fibrin Staabilization, and Fibrinolysis, J.G. Francis, Ed., pages 28, 31, VCH.) Consequently, it is entirely unlikely that monoclonals derived therefrom could have the precise specificity and sensitivity of the monoclonal used in the present invention and present claimed in new claim 22.

Rejections under 35 U.S.C. § 112 Second Paragraph

The Examiner has rejected claims 3-4 under 35 U.S.C. § 112 second paragraph stating that claims 3 and 4 are indefinite. The Applicants respectfully assert that this rejection is now moot in view of the Applicants' cancellation of claims 1-21. Moreover, new claims 22-27 now recite the sequence listing ID number for the claimed amino acid sequence and have amended the specification accordingly.

Rejections under 35 U.S.C. § 112 First Paragraph

The Examiner has rejected claims 1-5, 8 and 9 under 35 U.S.C. § 112 first paragraph stating that the present application is not enabling for methods of detecting cancer in subjects using any and all types of biological samples. The applicants respectfully traverse.

Claim 27 specifically limits the types of biological samples to the group consisting of blood, serum, plasma, urine, cervical secretions, bronchial aspirates, sputum, saliva, feces, synovial fluid and cerebrospinal fluid. Therefore, the Applicants are not claiming any on all types of biological samples. The Applicants are merely claiming biological samples from anatomical sites known to have cancer antigens associated with them.

The present invention comprises an immunoassay. Particularly an antigen capture assay. Traditionally antigen capture assays are notoriously robust and are used in a variety of clinical and environmental applications. The general principle of a capture assay involves coating a solid substrate with a reagent having a high affinity and avidity for an antigen sought to be detected. The capture reagent is most often an antibody, but recently, capture assays using aptamers, polynucleotides probes and other macromolecules have been developed. One of the most important aspects of capture assays is the versatility and adaptability of the assay principles to a wide range

of configurations. One of the classical configurations, and one of the embodiments of the present invention, is an antigen capture enzyme-linked immunoabsorbant assay (ELISA) in a 96 well micrometer plate format. In this format, the plastic microtiter plate wells are coated with an antibody, generally a monoclonal antibody, having a high affinity and avidity for the antigen to be detected. The coating procedure is well known to those of ordinary skill in the art and is routinely performed in laboratories daily throughout the world. After the capture antibody has been coated, the plastic surface is then "blocked' using a protein or other molecule composition that will not bind to the antigen to be detected. Blocking solutions are generally composed of protein solutions and the scientific literature is replete with sample solutions and many are available commercially. Next, a second antibody, generally a polyclonal antibody, that will also bind to the antigen to be detected with a relative high degree affinity and avidity is conjugated to a reporter molecule such as an enzyme. For example, horseradish peroxidase or alkaline phosphatase. The selection of the antibodies and the coating proceeds just described remains constant regardless of which biological sample is used as the test substrate. The nature of antibody-antigen interactions are well known to those of ordinary skill in the art. The biochemical environment that is optimum for each antigen-antibody reaction is easily determined using no more than routine experimentation by those having ordinary skill in the art of immunochemistry. Generally, the pH range, osmolarity and temperature that is optimum for a given antigen-antibody reaction falls into a very narrow range. Occasionally, pH, osmolarity and assay incubation time and temperature need to be adjusted to optimize the assay, however, these are minor adjustments that all immunochemists anticipate and compensate for when developing a new assay. Generally, and almost without exception, clinical samples such as the biological sources listed above, are diluted in a physiological buffer such as phosphate buffered saline having a physiological osomlarity and pH. The dilution factor is generally quite high due to the exquisite sensitivity of ELISA assays. Therefore, sample properties such as pH, protein content, and other interfering materials will be substantially diluted. Furthermore, all clinical laboratory technologists receive extensive training in sample preparation as part of their certification process. Moreover, it is routine for clinical laboratory technologists and

technicians to process clinical samples from ALL anatomical sites and they are specifically instructed and trained on assay system parameters and assay optimization.

Therefore, the Applicants respectfully assert that any experimentation necessary to optimize the assays of the present invention for use with the full range of clinical samples claimed in claim 27 is routine for any moderately skilled (i.e., even below the level of ordinary skill in the art) immunochemist or clinical laboratory technician.

The Examiner is directed to numerous publications and treatise that support the Applicants contentions. For example, see the Manual of Clinical Laboratory Immunology, an American Society for Microbiology publication, any edition. Specifically see the Fourth edition published in 1992 (well before the present application was filed). See sections A pages 1-61; section N pages 723 – 788; section at page 789 through 820. Although these sections describe assays ideally suited or the detection of antigens and antibodies in serum samples, the use of an antigen capture assays for non-serum-based antigen detection can be found at pages 616-617 where the authors describe using an antigen capture ELISA for detected viral antigens in crude biological samples. It can be seen by comparing the methodology describe at pages 616-617 with the methodologies decried for assays using serum samples that very little, if an differences exits between the assay configuration, preparation or performance.

Taken together, the cited reference demonstrates that experimental protocols for developing and optimizing immunoassays over a wide range of conditions is well known in the art and requires no more than ordinary skill and routine experimentation. Therefore, the Applicants respectfully assert that the Examiner's 35 U.S.C 112 first paragraph rejection has been traversed and its withdrawal is earnestly solicited.

Rejections under 35 U.S.C. § 102 (b)

The Examiner has rejected claims 1-5 and claims 8-10 under 35 U.S.C. § 102(b) as being anticipated by Koopman et al. (J. Lab. Clin. Med. Vol. 109, No. 1, 1987, page 75-84). The Applicants respectfully traverse this rejection. The Applicants have canceled claims 1-21 and have substituted new claims 22-27 therefore. Independent claim 1, the only independent claim recites a monoclonal antibody that binds to a fibrinogen degradation product (FDP) epitope of the beta chain of fibrinogen having an amino acid sequence corresponding to SEQ ID NO 1 and determining the presence or

absence of said FDP, wherein fibrin, fibrin degradation products and fibrinogen fragments D and E are not detected. Koopman et al. disclose an immunoassay using antibody, FDP-14, directed against a neoplastic determinant (E_{neo}) that, as described in more detail above, is found on the *gamma chain of fibrinogen*. The SEQ. ID. of claim 22 is located on the *beta chain of fibrinogen*. Therefore, Koopman does not possess all of the elements found in claim 22 and therefore cannot, by definition, anticipate the new claims. Consequently, the Applicants respectfully request that Examiner withdraw this basis for rejection.

The Examiner has also rejected claims 1-5 and claims 8-10 under 35 U.S.C. § 102(b) as being anticipated by McCulloch et al. (Haemostasis, Vol. 20, pages 73-80, 1990). The examiner asserts that because McCulloch et al. disclose the use of the Koopman immunoassay for the detection of cancer related antigens, that McCulloch anticipates cancelled claims 1-5 and claims 8-10 under 35 U.S.C. § 102(b). The Applicants respectfully traverse this rejection for the reasons stated immediately above and respectfully request that the Examiner withdraw this 35 U.S.C. § 102(b) rejection as well.

The Examiner has also stated that the Applicants must show an unobvious difference between the monoclonal antibody used by Koopman et al. and the monoclonal antibody used in the present invention (see Paper number 9 at page 8 line 3). The Applicants respectfully assert that the affinity and affinity of different monoclonal is inherently unpredictable and therefore one monoclonal cannot render another obvious merely because they may react with similar epitopes. However, in the present case the differences go beyond the non-obvious. The differences are stark. The monoclonal antibody used by Koopman reacts with the E fragment of fibrinogen to a significant degree. This is clearly illustrated at page 78 in Figure 2 of the Koopman et al. reference. As indicated above, fragment E does not contain a polypeptide with sequence GHRPLDK (Furlan, 1988). However, the monoclonal used in the present invention is non-reactive with the E fragment of fibrinogen as stated at page 12, line 22 of the present specification and illustrated by Figure 11c. Therefore, the Applicants respectfully assert that not only does the Koopman reference not anticipate the present

invention, it does not render it obvious and that any reference specifically using Koopman's immunoassay cannot anticipate or render obvious the present invention.

R jections und r 35 U.S.C. § 103

The Examiner has rejected claims 1-5 and 8-10 under 35 U.S.C. § 103 as being obvious over Amiral et al. (Blood Coag. Fibrinol., Vol. I, No. 4-5, pages 447-452, 1990) in view of Okajima et al. (Thrombosis Res. Vol. 66, pages 717-727, 1992) and Schwartz et al. (ACTA Medica Sustriaca, Vol. 8, No. 1, 1981). The Applicants respectfully traverse this basis of rejection for the following reasons.

The Examiner states that it would have been obvious to for one of ordinary skill in the art to modulate the method of Amiral et al. so as to include the detection of cancer because it was well known that fibrinogenolysis and fibrinogen degradation peptides were diagnostic of cancer. Moreover, the Examiner states that a person having ordinary skill in the art would have had a reasonable expectation of success is developing said method of detecting cancer in a subject because Amiral, at page 447 states "...primary fibrinolysis may be present and, in this case, only fibrinogen breakdown products are formed (FgDP). This concerns some malignancies such as prostate or meningioma." However, the Examiner acknowledges that the references do not specifically teach that the antibody(s) recognize epitope comprising SEQ.ID. NO. 1 of the present invention and has placed the burden on the Applicant to prove to the contrary. The Applicant respectfully asserts that they have met this burden as present in Table 1.

Amiral et al. describe both ELSA assays as well as latex agglutination assays using the antibodies, and combinations thereof described in his publication at page 449 column 1. The first ELISA immunoassay (A) comprised an immunoassay having significant fibrin degradation product (FbDP) detection activity with minimal, if any fibrinogen degradation product (FgDP) sensitivity. (See Amiral at pages 451 for details). The second ELISA assay (B) demonstrated good sensitivity for BOTH FbDP and FgDP, finally, the third ELISA assay (C) virtually specific for FbDP demonstrating no FgDP reactivity.

On page 449 at column two Amiral et al. describe latex agglutination assays made using the monoclonal antibodies previously described. The first latex

immunoassay used a monoclonal selected from Group 2. This assay demonstrated virtually complete sensitivity for FbDP (measured as D-dimmer) and was completely unreactive with D-monomer of FgDP or FgDP-D (although undefined by the authors, it is presumed the FgDP-D stands for fibrinogen degradation products. D-dimmer is generally associated with fibrin degradation; however, late stage degradation of fibrinogen can lead to D monomer production. Neither D-monomer nor D-dimmer contains GHRPLDK epitope in their β-chain. See: Jobe M.I. Mechanisms of Coagulation and Fibrinolysis. In: Steine-Martin, E.A., Lotspeich-Steininger, C.A., and Koepke, J.A., eds *Clinical Hematology*. Philadelphia: Lippcott, 1998;612-634.) The second latex agglutination assay described on page 449 used a combination of one Group 1 and one Group 3 monoclonal antibody. The resulting assay detected all degradation products whether associated with fibrin or fibrinogen.

Therefore, as can be seen by closely scrutinizing the Amiral reference, there are NO immunoassays disclosed that are specific for FgDP as claimed in the present invention. Moreover, a person having ordinary skill in the art would NOT have had a reasonable expectation of producing an assay having the specificity of the present invention using the technique disclosed in Amiral in combination with any of the cited references. First, Amiral does not suggest how one of ordinary skill in the art would make an immunoassay using monoclonal antibodies directed against fibrin and fibrinogen degradation products that would NOT also react with D-dimmer and D monomer. Furthermore, Amiral state at page 451 that D-dimmer assays are only useful for diagnosing thrombolytic tendencies or for monitoring thrombotic therapies. The authors go on to state that these assays are only valuable for detection fibrin degradation NOT fibrinogen degradation. The authors in Amiral et al. do not demonstrate the successful development of a single immunoassay, whether ELISA or latex agglutination that does NOT detect the D-dimmer. Therefore, persons seeking to develop assays that are specific for fibrinogen degradation that do not react with the D fragment as the assay presently claimed would not seek the guidance of Amiral et al. and therefore have no motivation to combine Amiral et al. with any other references.

Consequently, the Applicants respectfully assert that even though Amiral et al. does disclose a variety of monoclonal antibodies derived from animals immunized using

crude fibrin and fibrinogen degradation products and the development of a wide range of immunoassays using these antibodies, Amiral et al. does not provide any guidance relevant to the present invention. The fact that Amiral does not provide any teaching or suggestion of how to make a fibrinogen degradation product specific immunoassay renders it entirely unlikely that persons having ordinary skill in the art seeking to make fibrinogen degradation product specific assays would rely on this reference or be motivated to combined with others.

The Examiner has also cited Okajima et al. (Thrombosis Res. Vol. 66 pages 717-727, 1992 and Schwartz et al. (ACTA Medica Sustriaca, Vol 8, No. 1 1981) to buttress the teaching found in Amiral et al. However, neither Okajima et al. nor Schwartz et al. can make up the overwhelming deficiency of the primary reference, Amiral et al. As previously stated, Amiral's deficiency is that it fails to teach or suggest how one of ordinary skill in the art would make an immunoassay that detected fibrinogen degradation products and NOT D-dimmer and/or D monomer as enabled and claimed in the present application. Amiral et al. only describes immunoassays that detect BOTH fibrin D-dimmer and fibrinogen degradation products OR ONLY the D dimmer of fibrin.

Schwartz et al. discuss the clinical relevance of detecting fibrinogen degradation products as an aid to the diagnosis of cancer; however, Schwartz et al. does not provide any guidance on how one of ordinary skill in the art would make such an assay.

Okajima et al. describe in detail the clinical relevance of detecting soluble fibrinogen degradation products in samples taken from patients afflicted with metastatic prostrate cancer. However, the authors do not describe an immunoassay, nor even suggest an immunoassay that detects fibrinogen degradation products that do not also detect the D and E fragments of fibrinogen.

Okajima et al. discuss two types of immunoassays for the detection of fibrinogen degradation products. One assay is a latex agglutination assay described as using latex beads coated with a rabbit anti-human fibrinogen antibody. A second latex agglutination assay is described that measured plasma levels of cross-linked fibrin degradation products (XDP) using the JP-23 monoclonal antibody. As illustrated in Figures 1 and 3 in the cited reference, both the rabbit anti-human fibrinogen antibody, as well as the JP-23 monoclonal antibody are reactive with both the D and E fragments

of fibrinogen. Finally, an immunoblot assay is described at page 721 and the corresponding results are presented at page 722, Figure 1.

The immunoblot assay and one of the latex agglutination assays both utilize a rabbit anti-human fibrinogen antibody as described in the Methods and Materials Section at page 718. This antibody is further characterized at page 722 as shown in Figure 1. Figure 1 clearly demonstrates the antibody used to detect fibrinogen degradation products in the assays described in the Okajima et al. reference also detects D and E fragments in addition to DD dimmers and other early antigens including XY, XD and to some degree fibrinogen. Therefore, the Okajima et al. reference does not teach nor suggest how an assay specific for fibrinogen degradation products could be made that did not also detect D and E fragments as well as fibrinogen.

Consequently, taken together, Amiral et al., Okajima et al. and Schwartz et al. fail to teach all of the elements claimed in the present application. Furthermore, the Applicants respectfully assert that persons having ordinary skill in the art would not be motivated to combined these teachings because there is no teaching or suggestion that relates to an immunoassay that specifically detects fibrinogen degradation products with also detecting fragments D and E. Finally, the Applicants respectfully assert that the inability of all of the cited references to suggest a solution to the problem solved by the present inventors (development of an immunoassay that specifically detects fibrinogen degradation products without also detecting fibrinogen, fragments D and E) is demonstrative of the fact that persons having ordinary skill in the art would not have any reasonable expectation of success if these references were combined.

Therefore, in view of the arguments presented above, the Applicants respectfully request that the Examiner withdraw the 35 U.S.C. § 103 rejection presently of record.

Conclusion

The Applicants have presented arguments and evidence that support the assertion the specification as drafted is fully enabling for the full range of biological samples claimed. Moreover, the Applicants have demonstrated that the neither the Koopman et al. nor the McCulloch et al. reference anticipate the claims as presently drafted. Furthermore, the Applicants have provided arguments that support their

assertion that Amiral et al. does not render the present invention obvious regardless of what references of record it is combined with.

Therefore, the Applicants respectfully assert that all claims are allowable and therefore respectfully request that the Examiner allow all presently pending claims in this application. If the Examiner believes that a telephonic interview with the Applicants or the Applicants' attorney will advance the allowance of this case, please contact the undersigned at the telephone number provided below.

Attached hereto is a marked-up version of the changes made to the specification and claims by the current amendment. The attached page is captioned "**Version with markings to show changed made**."

The Commissioner is authorized to charge any fee which may be required in connection with this Amendment to deposit account No. 16-2230.

Respectfully submitted,

December 19, 2001

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Page 1, beginning at line 1:

This application claims the benefit of U.S. Provisional Application No. 60/048,405, entitled "IMMUNOASSAY FOR THE DETECTION OF CANCER," filed on June 3, 1997 (now abandoned) by Ngo et al., and U.S. Provisional Application No. 60/060,088 entitled "IMMUNOASSAY FOR THE DETECTION OF CANCER," filed on September 26, 1997 (now abandoned), by Ngo et al., which are incorporated by reference herein.

At page 11 at line 26:

In one embodiment of the present invention, the method measures proteolytic degradation of fibrinogen with minimal interference from intact fibrinogen. In this embodiment of the present invention, tow different antibodies are used as the detection system. One of the antibodies is specific for the peptide SEQ. ID. NO. 1: GHRPLDK which is part of the amino acid sequence of the β-chain of fibrinogen, located near its amino terminus.

At page 11 at line 32

Assay specificity is achieved by the use of two different antibodies in a two-site, solid-phase enzymometric assay. The more highly specific antibody, which is immobilized to the solid phase consists of a murine monoclonal to a glycine-histidine-arginine-proline-leucine-asparate-lysine-cycteine (SEQ. ID. NO. 2: GHRPLDKC) octapeptide. The first seven amino acids of this peptide represent an internal sequence within the β-chain of fibrinogen, which is near the amino terminus and is exposed after initial plasminolysis (residues 15-21). Chung et al., "Characterization of Complementary Deoxyribonucleic Acid and Genomic Deoxyribonucleic Acid for the β Chain of Human Fibrinogen," *Biochemistry*, 22:3244-3250, (1983). After capture of the proteolytic degradation products of fibrinogen by the immobilized monoclonal antibody, the immune complex is detected by using a highly specific conjugate consisting of polyclonal antifibrinogen antibody labeled with horseradish peroxidase.

At page 12 beginning at line 7

While the peptide SEQ. ID. NO. 1: GHRPLDK has been used in one mbodiment of the present invention, it will be clear to those skilled in the art that other internal fibrinogen peptides would also be of use, as would internal peptides of other proteins which are degraded by proteases produced by cancer. In an assay of the present invention a commercially available monoclonal antibody to the peptide SEQ. ID. NO. 2: GHRPLDKC can be used.

Please add the following new claims:

22. (New) A method for detecting cancer in a subject comprising:

contacting a biological sample obtained from said subject with a monoclonal antibody that binds to a fibrinogen degradation product (FDP) epitope of the beta chain of fibrinogen having an amino acid sequence corresponding to SEQ ID NO 1 and determining the presence or absence of said FDP, wherein fibrin, fibrinogen and fibrinogen fragments D and E are not detected.

23. (New) The method according to claim 22 wherein the step of determining the presence or absence of said FDP is performed using and enzyme-linked immunoadsorbent assay.

24. (New) The method according to claim 22 wherein said monoclonal antibody is generated using an immunogen prepared from a peptide having an amino acid sequence corresponding to SEQ. ID. NO. 2.

25. (New) The method according to claim 22 wherein said subject is a mammal.

26. (New) The method according to claim 25 wherein said animal is a human.

27. (New) The method according to claim 22 wherein in said biological sample is selected from the group consisting of blood, serum, plasma, urine, cervical secretions, bronchial aspirates, sputum, saliva, feces, synovial fluid and cerebrospinal fluid.

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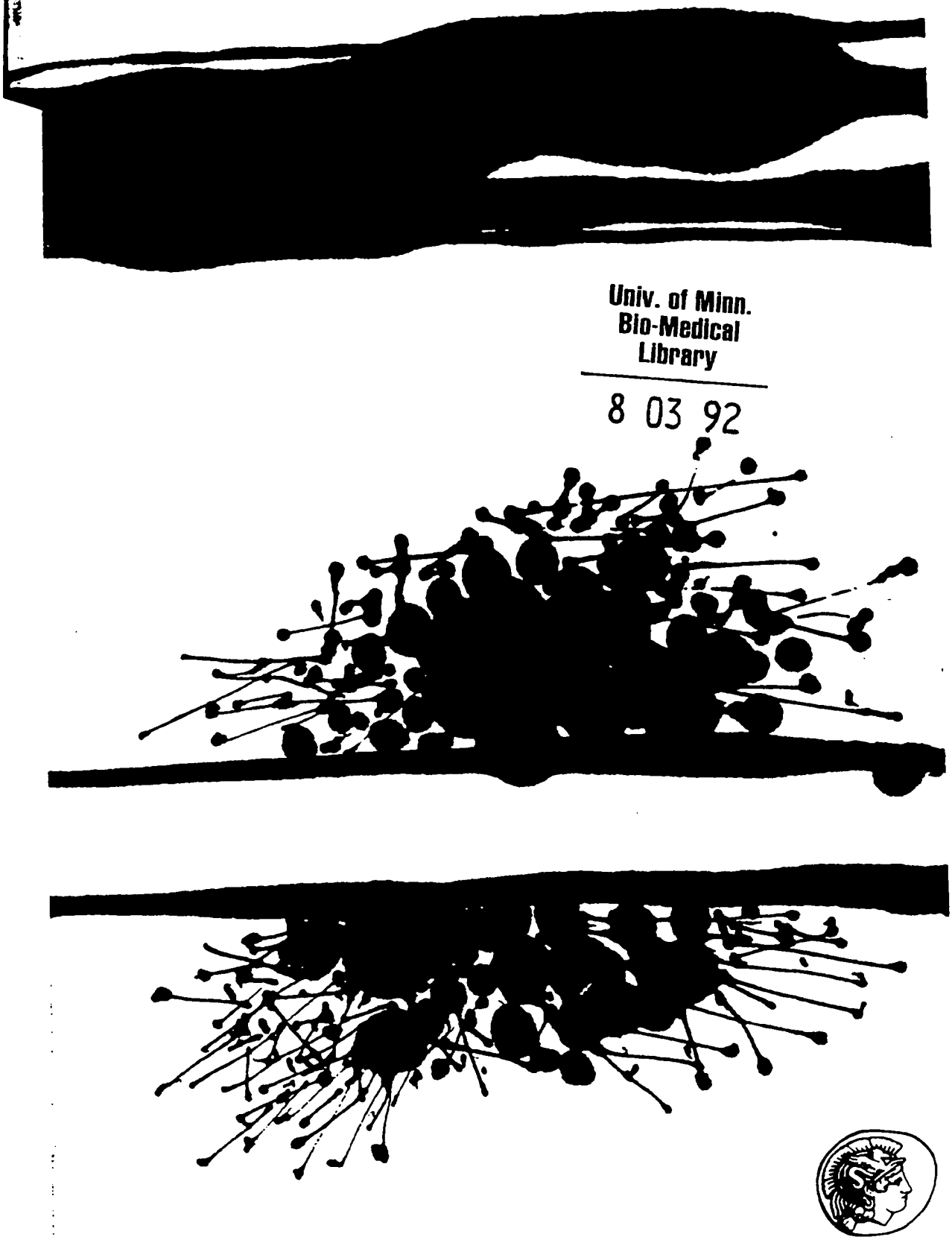

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DIRECT EVIDENCE FOR SYSTEMIC FIBRINOGENOLYSIS IN A PATIENT WITH METASTATIC PROSTATIC CANCER

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ABSTRACT Although the possible occurrence of systemic fibrinogenolysis has been suggested in patients with metastasising prostatic cancer (MPC), direct evidence is lacking. We report on a patient with MPC whose laboratory data were consistent with hyperfibrinolysis: marked decrease of α_2-antiplasmin (AP) level (less than 50% of normal), increase of plasmin-α_2-antiplasmin complex, D-fragment of fibrin and fibrinogen degradation products [FDP(D)] and cross-linked fibrin degradation products (XDP). The patient neither showed laboratory nor clinical evidence for consumption coagulopathy except for a slight increase in thrombin-antithrombin III complex level. Immunoblotting of the patient's serum using an anti-fibrinogen antibody revealed the presence of a 250 kDa protein in addition to DD fragments. Following reduction of this protein by 2-mercaptoethanol after extraction from SDS-PAGE gel, γ-chain of fibrinogen (47 kDa) was found by immunoblotting using a monoclonal antibody recognising a 86-302 residue of the γ-remnant of fibrinogen. Moreover, the 250 kDa protein did not bind to Sepharose 4B to which a monoclonal antibody recognising the N-terminus of fragment D was conjugated. These findings indicated that this protein was not fragment DY, but rather fibrinogen fragment X. With the retraction of the prostatic tumour by an effective therapy, the patient's AP level increased gradually. When the plasma AP level rose to 60% of normal, the fragment X was no longer detectable. These findings suggested that systemic fibrinogenolysis occurred in the patient with MPC only when AP levels were markedly decreased.

KEY WORDS: Fibrinogenolysis, prostatic cancer, α^2-antiplasmin

INTRODUCTION

The possible occurrence of systemic fibrinogenolysis has long been suggested in patients with MPC (1-3). However, since the distinction between fibrinogenolysis and fibrinolysis has been extremely difficult to determine by routine laboratory methods, direct evidence for fibrinogenolysis *in vivo* is lacking in patients with MPC. We have previously reported the critical importance of simultaneous determinations of XDP/FDP(D) ratios and AP levels for the evaluation of fibrinolysis in patients with disseminated intravascular coagulation (DIC) associated with acute promyelocytic leukemia (4). By following such criteria, we detected hyperfibrinolysis in a patient with MPC.

It was the aim of the present study to search for direct evidence for systemic fibrinogenolysis in this patient with MPC whose laboratory parameters indicated hyperfibrinolysis.

MATERIALS AND METHODS

Materials:

All chemicals were purchased from commercial sources. Thrombin and urokinase from Motida Pharmaceutical Co., Osaka, Japan; reptilase from Iatron Co., Tokyo, Japan; fibrinogen from Kabi Vitrum, Stockholm, Sweden; rabbit anti-human fibrinogen antibody, horseradish peroxidase (HRP)-conjugated rabbit anti-mouse immunoglobulin and HRP-conjugated rabbit anti-goat immunoglobulin from DAKO Inc., Glostrup, Denmark; alkaline phosphatase (ALP)-conjugated goat anti-mouse immunoglobulin, ALP-conjugated goat anti-rabbit immunoglobulin, and nitrocellulose membranes from BioRad, Richmond CA.; 5-Bromo-4-chloro-3-indolyl phosphate, nitro-blue tetrazolium and 3,3'-diaminobenzidine from Sigma Chemical Co., St. Louis, MO.; CNBr-activated Sepharose 4B from Pharmacia Fine Chemical, Uppsala, Sweden; molecular weight standards which included soybean trypsin inhibitor (21,500), carbonic anhydrase (31,000), ovalbumin (45,000), bovine serum albumin (66,200), phosphorylase b (92,500), ß-galactosidase (116,250) and myosin (200,000) were from BioRad. Monoclonal antibodies JIF-25 and JIF-23 were kindly supplied by Dr. Gilbu Soe, Research Division, Iatron Inc., Chiba, Japan. Purified fragment E (E1,2) was kindly supplied by Dr. Michio Matsuda, Division of Hemostasis and Thrombosis Research, Institute of Hematology, Jichi Medical School, Tochigi, Japan. Other reagents used were of analytical grade.

Patients' characteristics and blood collection

The patient, a 65-year male, was admitted to Kumamoto University Hospital for treatment of prostatic cancer with multiple bone metastases. Diagnosis of prostatic cancer was made by needle biopsy of his prostate tissue. The patient immediately underwent castration and hormonal therapy. Bleeding was not observed and, thus, antifibrinolytic agents were not used throughout his clinical course.

The patient's blood samples were collected with 1/10 volume of 3.8% sodium citrate and centrifuged at 2,000 x g for 10 min at 4°C. Plasma samples were stored at -70°C prior to laboratory analysis. To obtain the serum samples, plasma samples were incubated with 1/10 volume of saline containing 40 IU/ml bovine thrombin, 5mM $CaCl_2$ and 5.7 trypsin inhibitor units (TIU)/ml aprotinin for 30 min at room temperature and centrifuged at 15,000 x g for 30 min at 4°C. The resulting supernatant was used as serum sample. Blood samples for determination of FDP(D) fractions were collected into evacuated SPLI-tubes (Diagnostica Stago, France) which contained reptilase, aprotinin and calcium chloride (4). After 30 min at room temperature, blood samples were centrifuged at 2,000 x g for 20 min at room temperature, and the resulting supernatant fractions were used for FDP(D) determination.

Assays

FDP(D) fraction was determined by a latex agglutination method using anti-human fibrinogen antibody-coated particles in an automatic analyser LA-2000 (Eiken Chemical Co., Japan) as described (4). Plasma XDP levels were determined by a latex agglutination method using latex particles coated with a monoclonal antibody (JIF-23) against the plasmic fragment DD derived from human cross-linked fibrin (14). The measurement of XDP levels by latex agglutination was performed in an automatic analyser LPIA-100 (Iatron Co., Tokyo, Japan). AP levels were determined from the residual plasmin activity by using chromogenic substrate S-2251 (Kabi Diagnostica, Sweden) after incubation of plasma with excess plasmin as described (5). Soluble fibrin monomer complex (SFMC) was determined by a red cell agglutination method as described (6). Thrombin-antithrombin III complex (TAT) and plasmin-antiplasmin complex (PAP) were determined by enzyme immunoassay methods as described by Pelzer (7) and Harpel (8), respectively. Plasma levels of immunoreactive t-PA and u-PA were determined using commercially available kits (American Diagnostica Inc.).

Preparation and purification of the plasmic degradation products of fibrinogen and cross-linked fibrin

For purification of fragment D, human fibrinogen (12mg/ml) dissolved in 50mM sodium phosphate buffer, pH 7.5, containing 150mM NaCl, 5mM $CaCl_2$ and 0.05% NaN_3 was incubated with a mixture of 1.5 U/ml of plasminogen and 1,000 IU/ml of urokinase for 3 hours at 37°C. After addition of 100 TIU/ml of aprotinin, plasmic digests were gel filtered on a 2.6 x 67cm Sephacryl S300 (Pharmacia) in a buffer containing 50mM Tris-HCl, 28mM sodium citrate, 0.1M NaCl, 0.02% NaN_3 and 0.028 TIU/ml aprotinin, pH 7.4. The fractions containing D fragment were pooled and dialysed against 10mM Na_2HPO_4, pH 9.1, applied onto DEAE-cellulose column (Whatman, DE-52), 2.5 x 18cm, equilibrated with 10mM NaH_2PO_4, pH 9.1, followed by 320ml of 300mM KH_2PO_4, pH 4.4.

Preparation of cross-linked fibrin and its plasmic digest were performed according to the method of Olexa and Budzynski (9). Human fibrinogen (grade L, Kabi Vitrum) was enriched with factor XIII and clotted as described by Terukina (11). Cross-linked fibrin (1g) was suspended in 20ml of 0.15M Tris-HCl buffer, pH 7.8, containing 5mM $CaCl_2$ and 0.02% NaN_3. Plasmin (0.53 IU/ml) was added to this solution and incubated for 2 hours at 37°C. At the end of incubation, 500μl of 100 TIU/ml aprotinin was added to terminate the reaction. Plasmic digests of cross-linked fibrin were then applied to a lysine Sepharose 4B column (Pharmacia) to remove plasmin. Plasmic digests of cross-linked fibrin were used as standard FDP (STFDP) in the experiments. STFDP was then gel filtered on Sephacryl S300 (Pharmacia) to separate FDP fragments with high molecular weight (fragments with molecular weight higher than that of DY/YD or YY/DXD) from those with low molecular weight (mainly containing DD/E). The high molecular weight FDP fragments [FDP(A)] and the low molecular weight FDP fragments [FDP(B)] were used as standard materials in the experiment. For purification of DD/E, FDP(B) was rechromatographed on Sephacryl S300 to remove contaminating high molecular weight FDP fragments.

SDS polyacrylamide gel electrophoresis and protein extraction

SDS polyacrylamide gel electrophoresis (SDS-PAGE) with 4% and 10% gel or 3.5% to 9% linear gradient gel in 0.1% SDS were performed as described (10). Gels were silver-stained using a commercially available kit (Daiichi Pure Chemicals Co., Tokyo, Japan).

After SDS-PAGE, gels between 2.5cm and 7.5cm from the upper edge were cut at 5mm widths. Protein was extracted by incubating each gel fragment with a solution of 0.02M Tris-HCl, 0.15M NaCl and 0.1% SDS, pH 7.6, for 72 hours at 25°C and the solubilised protein was then precipitated with acetone. The precipitate was

evaporated to dryness and dissolved in buffer solution containing 0.0625M Tris-HCl (pH 6.8), 1.8% SDS, 6.4% glycerol, 0.005% bromphenol blue with or without 0.58M 2-mercaptoethanol (2-ME). The samples thus btained were subjected to SDS-PAGE and immunoblotting.

Immunoblotting

For the separation and identification of degradation products derived from fibrin and fibrinogen, immunoblotting was performed using a rabbit anti-human fibrinogen antibody (Dako Inc.) or monoclonal antibody against γ-remnant (86-302 residue segment) of fibrinogen (JIF-25) (11) as first antibodies. After separation by SDS-PAGE, proteins were electrophoretically transferred from the gel onto nitrocellulose at 20mA/cm^2 of nitrocellulose membrane for 2 hours using an electroblot apparatus (Holizeblot AE-6670, ATTO Co. Tokyo) according to the method described by Towbin et al. (12). The transfer buffer consisted of 0.1M Tris-HCl, pH 8.3, containing 0.192M glycine, 20% methanol and 0.02% SDS. After transfer, the nitrocellulose membranes were soaked in 0.02M Tris-HCl and 0.15M NaCl (pH 7.6), containing 1% (w/v) gelatin for 1 hour at room temperature and incubated for 3 hours at room temperature with either 50μg/ml rabbit anti-fibrinogen antibody or JIF-25 in the same buffer. After three wash cycles with 0.02M Tris-HCl and 0.15M NaCl, containing 0.1% Tween 20, pH 7.6, the membranes were incubated for 1 hour at room temperature with alkaline phsophatase-conjugated goat anti-rabbit IgG or anti-mouse IgG (BioRad Inc.). They were finally washed 4 times with the buffer and incubated with 100mM 5-bromo-4-chloro-3-indolyl phosphate and 100mM Nitro-blue tetrazolium in 100mM diethanolamine, 5mM $MgCl_2$ buffer, pH 9.5, for colour development.

Adsorption of the serum sample by immuno-affinity gel

$F(ab')_2$ fragment of monoclonal antibody (JIF-23) was coupled to CNBr-activated Sepharose 4B according to the manufacturer's instruction. Ten μl of the serum sample was incubated with 200μl of the gel suspension (50% v/v) in 0.05M Tris-HCl, pH 8.0, and 0.15M NaCl for 13 hours at 4°C. The mixture was then centrifuged at 10,000 x g for 10 min at 20°C. The resulting precipitate was resuspended and subjected to 3 cycles of incubation in 0.02M Tris-HCl, pH 7.6, 150mM NaCl and 0.05% Tween-20 for 30 min at 25°C, followed by centrifugation at 10,000 x g for 10 min at 20°C. The precipitate was incubated with 0.02M Tris HCl, 150mM NaCl, and 2% SDS, pH 7.6, followed by centrifugation at 15,000 x g for 10 min at 20°C. Protein in the supernatant was concentrated by acetone precipitation and subjected to SDS-PAGE.

Immunohistochemical staining

Staining of t-PA: 4μm sections were cut from a paraffin tissue block, deparaffinized by xylene and ethanol and treated with 1% H_2O_2 for 5 min. Sections were treated for 30 min with normal goat serum followed by a 1 hour incubation with 10μg/ml goat anti-t-PA antibody, washed with phosphate buffered saline (PBS) and incubated with horseradish peroxidase (HRP)-labelled anti-goat immunoglobulin (Dako Inc.; 1/100) for 1 hour. After another PBS wash cycle, HRP enzyme colour development was effected with 3,3 diaminobenzidine, 3% H_2O_2 in PBS. All incubations were done at room temperature. Staining of u-PA was performed according to the same method as described above except that anti-u-PA monoclonal antibody (# 394, American Diagnostica Inc.; 10μg/ml), mouse immunoglobulin (IgG) and HRP-labelled rabbit anti-mouse IgG (Dako Inc.; 1/100) were used.

RESULTS

Coagulation and fibrinolysis in the patient with MPC

Since DIC is frequently associated with MPC (13), laboratory examination of coagulation and fibrinolysis parameters was performed (Table 1). Plasma level of AP markedly decreased (less than 50% of normal) and plasma PAP level was elevated.

Although plasma FDP level was remarkably increased, XDP was only slightly el - vated. XDP/FDP(D) rati was thus decreased in the patient. There was no laboratory or clinical evidence for DIC in the patient except a slight increase in TAT value. Liver function was normal in the patient. Plasma levels of t-PA and factor XIII were within the normal ranges and u-PA was not detectable in the patient. These findings strongly suggested hyperfibrinolysis in the patient.

Table 1 Laboratory findings of the case with metastasising prostatic cancer (MPC)

Tests	MPC	normal range
SFMC	(-)	(-)
Fibrinogen	618mg/dl	144-496
TAT	7.6ng/ml	-5.0
FDP(D)	20,390ng/ml	44-496
XDP	3.32μg/ml	-0.5
AP	36%	90-120
PAP	2.6μg/ml	-0.8
t-PA	4.0ng/ml	
u-PA	below test range	

Analysis of FDP by immunoblotting

For the more detailed characterisation of the fibrinolytic state, immunoblotting of the patient's serum samples was performed using anti-human fibrinogen antibody. As shown in Fig. 1, in addition to DD, bands corresponding to fragments X or DY (M_r 250 kDa) were detected in the patient's serum. After SDS-PAGE of the patient's serum sample, the 250 kDa protein was extracted from the gel with 0.1% SDS. The extracted protein revealed a single band upon immunoblotting using anti-human fibrinogen antibody (data not shown). The extracted protein was reduced with 2-ME and analysed by immunoblotting using monoclonal antibody against the γ-remnant of fibrinogen (JIF-25) (11). As shown in Fig. 2, γ-chain of fibrinogen (M_r 47 kD) was demonstrated after a reduction of the 250 kDa protein. γ-γ Dimer was detected after a reduction of the standard DY fragments (Fig. 1). These findings indicated that the band corresponding to the 250 kD protein in the MPC patient's serum sample was not fragment DY, but fragment X, and further indicated fibrinogenolysis in addition to fibrinolysis in this patient.

Adsorption of the serum sample by monoclonal antibody (JIF 23)-conjugated Sepharose 4B

To confirm that the 250 kDa protein was not fragment DY but rather fragment X in the patient's serum, reactivity of the protein with monoclonal antibody recognising N-terminus of fragment D (JIF 23) (14) was examined. After incubation of the serum sample or the various standard samples of FDP with JIF 23-conjugated Sepharose 4B, the protein was eluted by buffer solution containing 2% SDS. The eluted protein was then analysed by SDS-PAGE. As shown in Fig. 3, the 250 kDa protein in the patient's serum was not detected in the eluted fraction, while standard DY fragment was detected. These results indicated that the protein with M_r 250 kDa in the patient's serum did not react with JIF 23, suggesting that the protein was not fragment DY, but fragment X.

Changes in the levels of AP, FDP(D), XDP, PAP and the fragment X in the clinical course

Behaviour of AP, FDP(D), XDP, PAP and the fragment X during the clinical course was shown in Fig. 4. The prostatic tumour gradually retracted following castration and hormonal therapy as judged by the changes in the acid phosphatase serum levels. Plasma levels of FDP(D) and PAP decreased over the clinical course with a concomitant increase in AP levels. Although plasma levels of FDP(D) decreased remarkably, those of XDP did not significantly change throughout the clinical course. Fragment X was detected in the patient from Day 1 (Day 1 is the day of

admission) to Day 10 of his clinical course and disappeared after Day 12. These findings suggested that the major part of the patient's FDP could be explained by fibrinogen degradation products (fragment X) and that fibrinogenolysis occurred only when the plasma levels of AP decreased markedly. These data also suggested that fibrinogenolysis might be intrinsically related to the presence of the prostatic tumour.

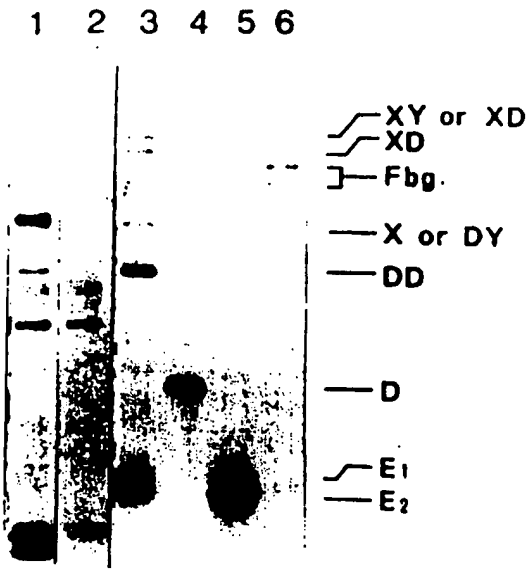

Fig. 1: Immunoblotting of FDP in the patient's serum using anti-human fibrinogen antibody.
After SDS-PAGE with 3.5% to 9% linear gradient gel, serum samples from the patient with MPC and a normal human subject were analysed by immunoblotting using a rabbit anti-human fibrinogen antibody as described in Materials and Methods. Lanes 1-2 are for serum samples and lanes 3-6 are for standard materials. Lane 1, the serum sample from the patient with MPC; lane 2, serum sample from a normal human subject; lane 3, standard FDP preparation; lane 4, fragment D; lane 5, fragment E; lane 6, fibrinogen.

Immunohistochemical staining of u-PA and t-PA in the prostatic tumour cells
Many authors report the occurrence of plasminogen activator in neoplasia (15). Thus, we considered that fibrinogenolysis in the patient might be induced by plasminogen activators produced by the prostatic tumour cells. To examine this possibility, localisation of t-PA and u-PA in the prostatic tumour cells were analysed immunohistochemically using the tumour tissue obtained by needle biopsy. u-PA was not demonstrated in tumour cells by immunohistochemical staining, while t-PA was demonstrated intracellular in many tumour cells (data not shown). These findings strongly suggested that the fibrinogenolysis observed in the patient could be induced by t-PA which was produced by the prostatic tumour cells. This finding is consistent with the finding that t-PA was demonstrated in the patient's plasma while u-PA was not.

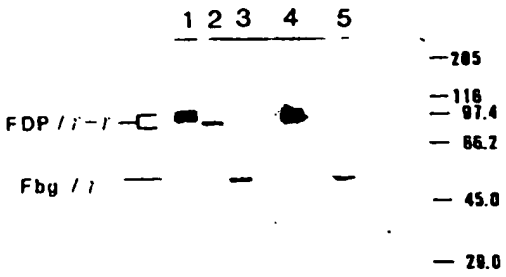

Fig. 2: Immunoblotting of the protein with M_r 250 kDa and fragment DY after reduction with 2-mercaptoethanol using monoclonal antibody JIF-25.
After SDS-PAGE with 3.5% - 9% linear gradient gel, the protein with M_r 250 kDa in the patient's serum samples and fragment DY in STFDP were extracted from the gel. The extracted protein with M_r 250 kDa and DY fragment were reduced with 2-mercaptoethanol. The resultant metabolites were analysed by immunoblotting using monoclonal antibody JIF-25 as described in Materials and Methods. Immunoblotting of FDP (A) (lane 1), FDP (B) (lane 2), fibrinogen (lane 3) extracted DY fragment after reduction (lane 4) and the extracted protein with M_r 250 kDa after reduction (lane 5) were shown. M_r (kDa) values are also indicated.

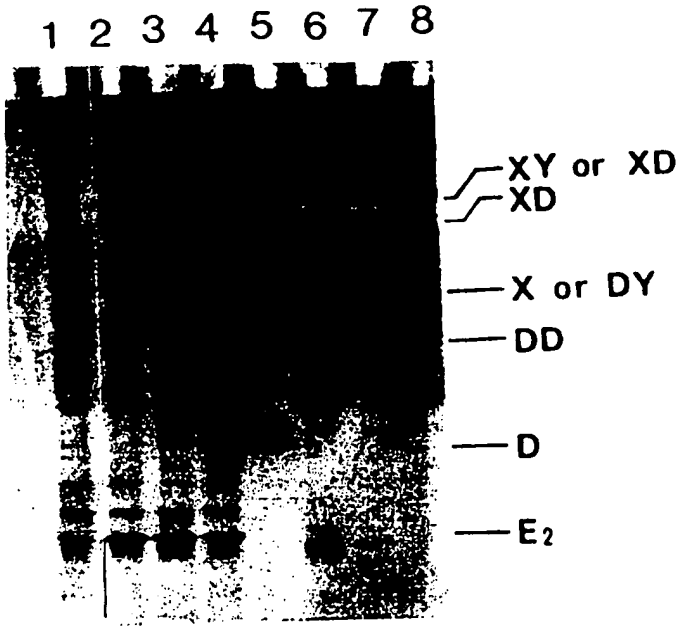

Fig. 3: SDS-PAGE of patient's FDP bound to Sepharose 4B-conjugated monoclonal antibody JIF 23.
Serum sample of the patient with MPC, STFDP, fragment DD/E and D were incubated with Sepharose 4B to which monoclonal antibody JIF-23 was conjugated. The gels were then treated with 0.2% SDS to dissociate the bound proteins as described in Materials and Methods. Recovered proteins derived from the serum sample of the patient with MPC (lane 1), standard preparation of FDP (lane 2), fragment DD/E (lane 3) and fragment D (lane 4) were analysed by SDS-PAGE with 10% gel according to the method of Laemmli (10). SDS-PAGE of fragment D (lane 5), fragment E_2 (lane 6), DD/E (lane 7) and STFDP (lane 8) were also shown.

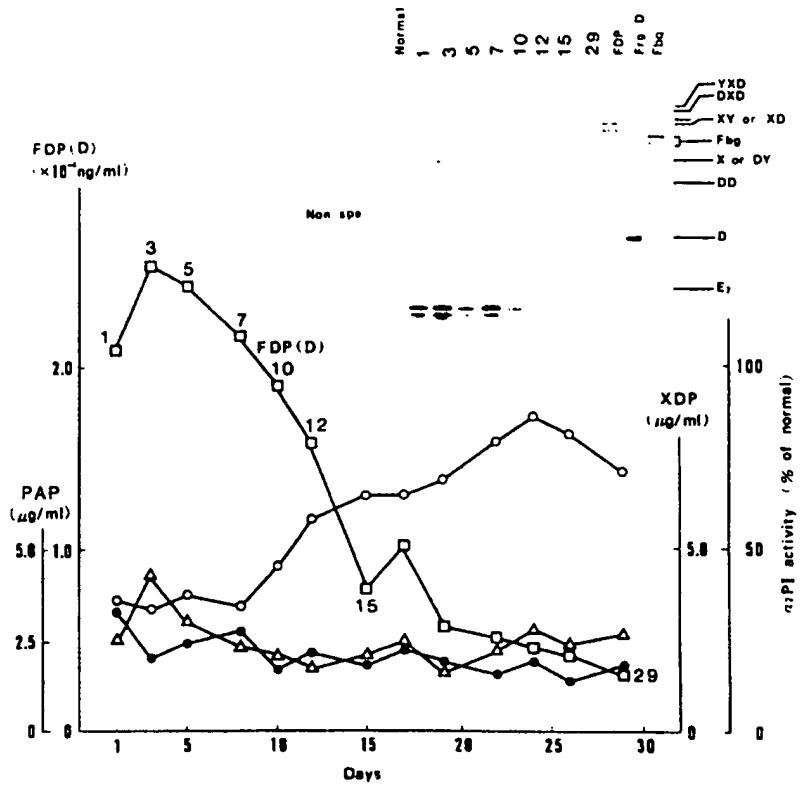

Fig. 4: Changes in the plasma levels of FDP(D), XDP, AP, PAP and fragment X in the clinical course of the patient with MPC.
Plasma levels of FDP(D) (□), XDP (●), AP (○) and PAP (△) were determined during the clinical course of the patient with MPC as described in Materials and Methods. Inset shows the immunoblotting of the serum samples and standard materials of FDP, fragment D (Frg. D) and fibrinogen (Fbg) using anti-human fibrinogen antibody. Numbers in the figure and inset represent day of his clinical course. Immunoblotting was performed as described in Materials and Methods.

DISCUSSION

Although fibrinogenolysis is a very rare pathological condition, its occurrence in patients with MPC has been suggested previously (1, 2). For the following reasons it is difficult to distinguish between fibrinolysis and fibrinogenolysis: (1) measurements of FDP by latex particle agglutination assay or hemagglutination assay using anti-fibrinogen antibody do not distinguish between the degradation products of fibrin and fibrinogen, (2) MPC is frequently associated with DIC (13) in which fibrinogen decreases due to the action of thrombin and FDP increases as a result of secondary fibrinolysis. Thus, direct evidence for fibrinogenolysis is often lacking in patients with MPC. Tagnon et al (1) showed the occurrence of hyperfibrinolysis in patients with MPC based on the findings that the plasma from such patients accelerated the degradation of fibrin *in vitro*. Booth and Bennett (3) detected PAP but not TAT, in the plasma of a patient with bleeding disorders suffering from MPC by crossed-immunoelectrophoresis. However, direct evidence for the fibrinogen break

down was not shown in these reports. Fibrinogenolysis has been clearly demonstrated in patients with pulmonary embolism who received thrombolytic agents and patients with acute myocardial infarction by a combined use of SDS-PAGE, radiolabelled anti-fibrinogen antibody and autoradiography (16). As could be shown in this report, immunoblotting is a useful tool to distinguish between the degradation products of fibrin and those of fibrinogen.

Interestingly, the fibrinogen degradation product (fragment X) was observed only when plasma levels of AP decreased to less than 60% of normal with a simultaneous decrease of XDP/FDP(D) ratio. These findings are consistent with the notion that the decrease of AP levels to less than 60% of normal might be a prerequisite for the occurrence of hyperfibrinolysis as suggested by Collen and Verstraete (17) and Hayashi and Yamada (18). We also demonstrated that the concurrent decrease of AP and the XDP/FDP(D) ratio might be the characteristic findings for the occurrence of fibrinogenolysis in the patients with acute promyelocytic leukemia (4).

It is important to distinguish between fibrinolysis and fibrinogenolysis in patients with DIC, since anti-fibrinolytic therapy should be considered only in DIC patients who showed evidence of fibrinogenolysis. Thus, it is important to determine simultaneously the level of AP and XDP/FDP ratio for the evaluation of the fibrinolytic state. However, the difference between FDP(D) and XDP could reflect not only fibrinogen degradation products but also the degradation products derived from non-crosslinked fibrin, especially in a thrombotic state (19). Preliminary immunoblotting using anti-fibrinogen antibody in another MPC patient with DIC demonstrated fragment D in addition to fragments DY and DD. However, fragment D could only be detected when the plasma levels of AP and XDP/FDP(D) ratio decreased markedly throughout his clinical course. Whether the D fragment detected in this patient might be derived from fibrinogen or non-crosslinked fibrin is not known. However, anti-fibrinolytic therapy as well as anticoagulant therapy should be considered in such a case since the pathological conditions in such a case would be similar to that in factor XIII deficiency.

Numerous reports have demonstrated the occurrence of plasminogen activators in cancer cells. Most of plasminogen activators found in cancer tissue or many culture cell types of neoplastic origin were u-PA. The role of u-PA in the spread of malignant cells has been extensively discussed (15). Although t-PA has not been directly implicated in the proliferation and spread of tumour cells, the occurrence of t-PA has been demonstrated in several cell lines of neoplastic origin most notably melanomas (20), colon cancer (21), breast (22) and prostate carcinoma (23). However, only a single report in the literature (24) has shown that t-PA can also be histochemically revealed in prostatic cancer tissue. Demonstration of the localisation of t-PA in the prostatic tumour cells by immunohistochemical staining in the MPC patient raises the possibility that t-PA derived from the prostatic tumour cells might contribute to fibrinogenolysis.

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